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Short communication

Features of basal and race-specific defences in photosynthetic *Arabidopsis thaliana* suspension cultured cells

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SUMMARY

Plant suspension cell cultures display many features of the innate immune responses observed *in planta* and have been extensively applied to the study of basal and race-specific defences. However, no single model including photosynthetic cultured cells has been used for the exhaustive characterization of both basal and race-specific defences to date. In this article, we report the activation of basal and race-specific defences in green cultured cells from *Arabidopsis thaliana*. Inoculation of cultured cells with isogenic virulent or avirulent strains of *Pseudomonas syringae* pv. *tomato* DC3000 (Pst) was used to evaluate race-specific defences. The proliferation of avirulent Pst was found to be lower than that of virulent Pst in the inoculated cultures. Extracellular pH changes, sustained oxidative burst (5–13 h post-inoculation), enhancement of salicylic acid, and massive cell death were specifically stimulated by the avirulent bacterium. Neither avirulent nor virulent Pst induced markers of basal resistance, such as callose deposition or early oxidative burst (1–5 h post-inoculation). However, both basal defences were activated when cells were exposed to *Pseudomonas syringae* pv. *phaseolicola* or to the Pst mutant defective in the type III secretion system (TTSS), Pst-hrpL$^-$. Thus, in these cells, basal defences may be inhibited by Pst in a TTSS-dependent manner. Recapitulation of classical defence features demonstrates the usefulness of this system for the fine characterization of plant innate immune components.

Plants counteract the attack of microbial pathogens by activating an innate immune system, including mechanisms of basal and race-specific resistance (Jones and Dangl, 2006). Basal resistance is founded on the recognition of non-self components (microbe-associated molecular patterns) and accounts for the general reactions in response to pathogen-derived molecules, including those from non-pathogenic microorganisms (Bent and Mackey, 2007; Zipfel, 2008). The induction of basal resistance involves mitogen-activated protein kinase signalling, Ca^{2+} and H^+ influx, early accumulation of reactive oxygen species (ROS), cell wall thickening leading to papillae formation, and altered expression of pathogen-responsive genes (Ausubel, 2005; Chisholm *et al.*, 2006; Lu *et al.*, 2001). Virulent races of pathogens can overcome basal resistance using diverse strategies, including the secretion of effector proteins suppressing defences into the plant cell (Alfano and Collmer 2004; Chisholm *et al.*, 2006; Hauck *et al.*, 2003; Jones and Dangl, 2006; Kim *et al.*, 2005; Speth *et al.*, 2007). Plants have evolved disease resistance proteins (R) that recognize pathogen effectors, often in an indirect manner, and elicit the so-called race-specific resistance (Bent and Mackey, 2007; Chisholm *et al.*, 2006; Jones and Dangl, 2006). The induction of race-specific resistance usually leads to the hypersensitive response (HR), a reaction leading to the cell death of challenged tissues, which contributes to the limitation of the propagation of pathogens as well as to the signalling of resistance in uninfected tissues (Greenberg and Yao, 2004). HR activation involves an increase in cytosolic Ca^{2+}, H^+ influx and extracellular alkalinization, activation of protein kinases, accumulation of ROS, nitric oxide and salicylic acid (SA), and, in contrast with basal resistance, triggers cell death (Greenberg and Yao, 2004; Lamb and Dixon, 1997; Mur *et al.*, 2008). Many forms of HR are light dependent and chloroplasts may contribute to ROS generation in HR (Mur *et al.*, 2008). Moreover, chloroplasts are targets of some pathogenic bacterial effectors (Jelenska *et al.*, 2007).

The identity, roles and networks among the defence components have been widely characterized in different experimental systems. The restriction of pathogen proliferation results from the successful spatial and temporal coordination of responses induced by both infected cells and surrounding healthy tissues. Thus, whole plant systems represent integral models for comprehensive studies on plant defences, whereas single plant cultured cells constitute simplified systems for the evaluation of

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biochemistry and signalling events of responses induced by challenged cells. Cultured cells from tobacco, tomato, soybean, bean, rose, rice and *Arabidopsis*, in particular, have contributed to the definition of the early kinetics of defences as, unlike whole plants, they allow the examination of rapid and homogeneous responses after synchronized elicitation (Atkinson *et al.*, 1985; Bolwell *et al.*, 1998; Davies *et al.*, 2006; Felix *et al.*, 1999; Glazener *et al.*, 1996; Jabs *et al.*, 1997; Krause and Durner, 2004; Lacomme and Roby, 1996; Lamb and Dixon, 1997, and references therein; Levine *et al.*, 1994). However, none of these *in vitro* systems provided a detailed examination of both basal and race-specific resistance features. Moreover, several *in vitro* systems employed to evaluate HR components involve etiolated cells. Thus, *in vitro* model systems have been proven to be limited for studies on interactions between defence mechanisms, as well as for the full characterization of light-sensitive HR components.

Roby and colleagues have successfully employed *Arabidopsis thaliana* Col-0 photosynthetic cultured cells to isolate genes with differential responses to non-isogenic virulent and avirulent races of *Xanthomonas campestris* pv. *campestris* (Daniel *et al.*, 1999; Lacomme and Roby, 1996, 1999). However, the full characterization of defences triggered by these cells has not been reported.

To contribute to this, in this article, we characterize the features of basal and race-specific defences activated by these *Arabidopsis* cells. Race-specific defences triggered by AvrRpm1 were evaluated using isogenic virulent (Pst-vir) and avirulent (Pst-AvrRpm1) races of *Pseudomonas syringae* pv. *tomato* DC3000 (Pst). Basal defences were analysed in response to the non-pathogenic bacterium *Pseudomonas syringae* pv. *phaseolicola* NPS3121 (Psp), or the Pst mutant defective in the type III secretion system (TTSS), Pst-hrpL$^-$. The effects of these pathogens on *Arabidopsis* tissues have been analysed extensively (Ham *et al.*, 2007; Hauck *et al.*, 2003; Kim *et al.*, 2005; Lindeberg *et al.*, 2006; Lu *et al.*, 2001; de Torres *et al.* 2003).

Our results indicated that features of basal and race-specific defences induced by *Arabidopsis* cultured cells were highly similar to those activated by plant tissues. This model plant–pathogen system can contribute to overcome the limitations previously described for the study of plant defence components *in vitro*.

Suspension cultures from *Arabidopsis thaliana* Col-0 photosynthetic cells were provided by Dr Roby (LIPM, Castanet-Tolosan, France). Cells were subcultured every 7 days and maintained under continuous cool-white fluorescent light (Lacomme and Roby, 1996) (45–55 μmol photons/m^2 s); they were treated with bacteria at day 6 after subculture, when the packed cell volume had reached 25% (v/v). Cultured cells showed 89.5%–95.9% viability during 7 days after subculturing (Fig. S1, see 'Supporting Information'). All bacterial strains were grown overnight in King's B medium [up to about 2×10^9 colony-forming units (cfu)/mL]; they were then washed and pre-incubated in minimal medium (3% sucrose, 100 mM K_2HPO_4, pH 5.8; 2 h, 20 °C) to promote TTSS functions (Yucel *et al.*, 1989). These bacterial suspensions were inoculated at a final concentration of 10^8 cfu/mL.

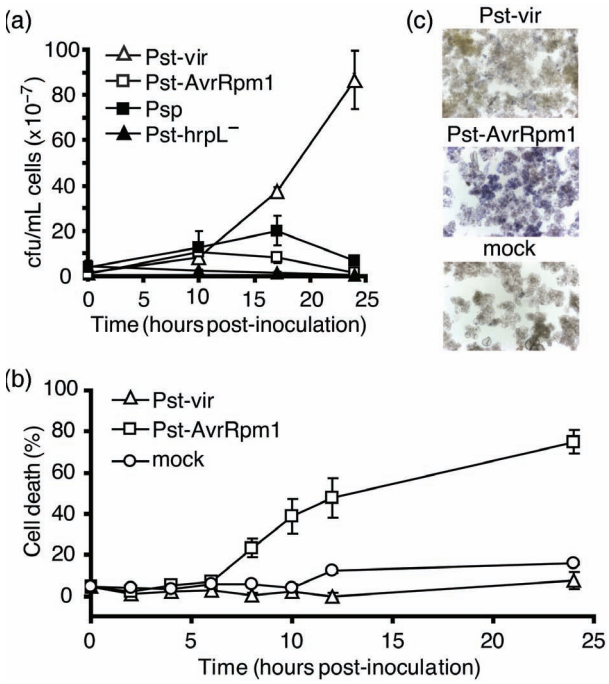

Fig. 1 Bacterial proliferation and cell death stimulation in *Arabidopsis* cultured cells inoculated with pathogens. Bacterial suspensions were treated as indicated in the text prior to their inoculation. Inoculations were performed with minimal medium (mock), *Pseudomonas syringae* pv. *tomato* virulent (Pst-vir), avirulent (Pst-AvrRpm1) or mutant strain defective in the type III secretion system (TTSS) (Pst-hrpL$^-$), as well as with *Pseudomonas syringae* pv. *phaseolicola* (Psp). (a) Bacterial growth was determined from aliquots of inoculated cultures isolated at the indicated times. To dissociate bacteria from plant cell clusters, 1-mL aliquots were placed into 1.5-mL Eppendorff tubes, disrupted using blue plastic pestles (three times, 1 min each), and then diluted and processed as described previously (Alvarez *et al.*, 1998). Data are represented as the mean ± standard deviation (SD) of one representative experiment from four independent experiments. (b) Cell death was assayed by Evans blue staining as described previously (Levine *et al.*, 1994). The 100% cell death value corresponds to that detected in heat-killed cells (65 °C, 15 min) from 6-day-old untreated cultures. Data are represented as the mean ± SD from four independent experiments. (c) Samples from inoculated cultures isolated at 24 h post-inoculation and stained with Evans blue were analysed by light microscopy.

To evaluate the features of race-specific resistance, we treated cultured cells with Pst-vir or Pst-AvrRpm1 (for bacterial strains, see Fabro *et al.*, 2004). Bacterial growth was assessed in these cultures at 10, 17 and 24 h post-inoculation (hpi) (Fig. 1a). In four different experiments, we observed that, from 17 hpi, the population of Pst-AvrRpm1 was lower than that of Pst-vir, and, at 24 hpi, the former pathogen reached 8–60-fold lower titres than the latter.

As markers for race-specific resistance, we evaluated extracellular alkalinization, ROS and SA production, the expression of ROS- and SA-sensitive genes and cell death in the inoculated cultures.

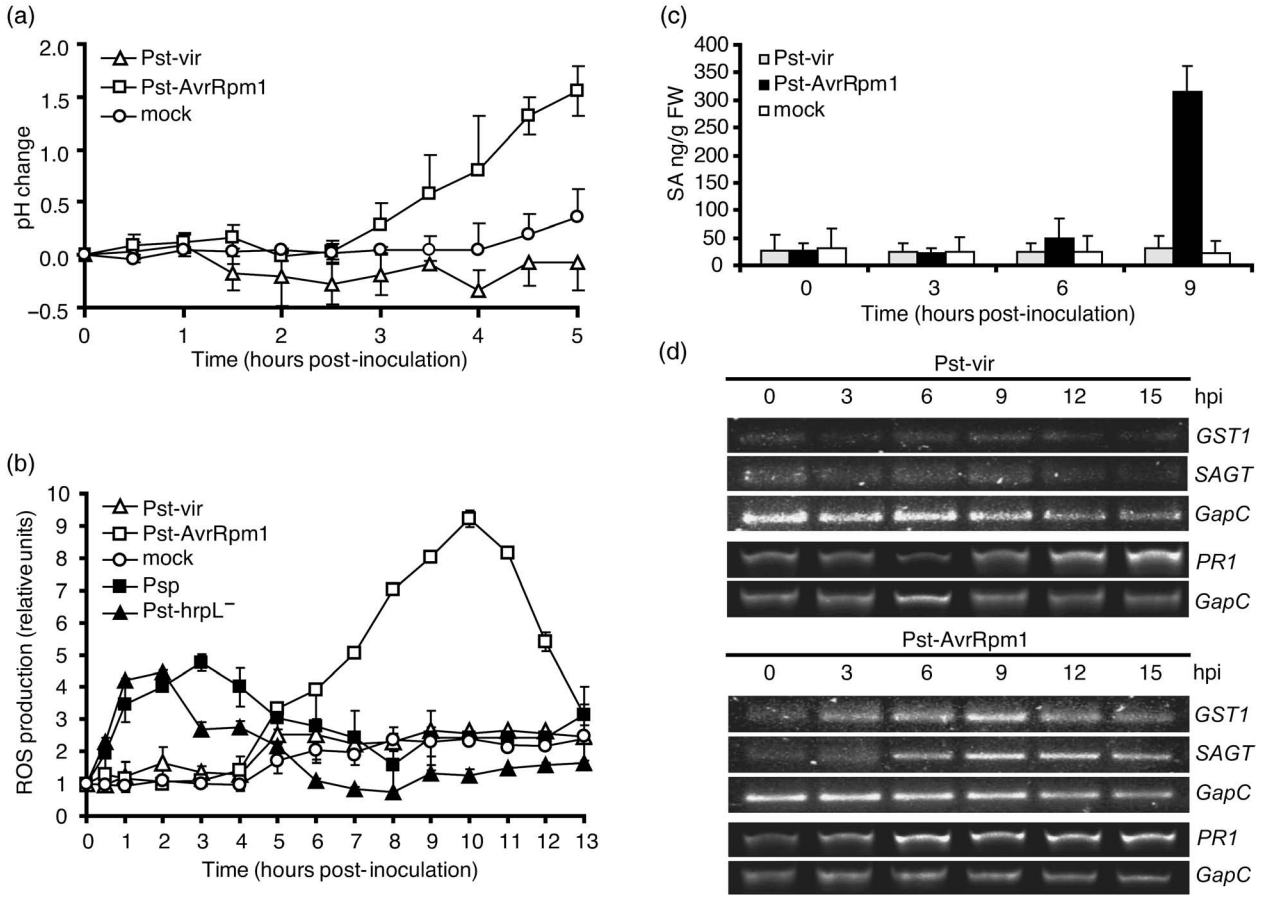

Fig. 2 Features of race-specific defence in *Arabidopsis* cultured cells. Inoculations were performed as indicated in Fig. 1. (a) The pH of the extracellular medium was determined in the supernatant of inoculated cultures. (b) Reactive oxygen species (ROS) levels were determined by 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) staining (Molecular Probes; final concentration, 10 μM) in 1-mL aliquots of inoculated cultures (Krause and Durner, 2004). The fluorescence intensity was measured in multiwell plates, exactly 5 min after probe addition, using a Fujifilm (Japan) FLA-3000 Fluorescence and Storage PhosphorImager. Data are represented as the mean ± standard deviation (SD) of one representative experiment from four independent experiments. (c) Total salicylic acid (SA) levels were determined in 1-mL aliquots of inoculated cultures. Suspension cultures were centrifuged to pellet cells, which were lyophilized and used for hormone determinations (Verberne *et al.*, 2002). Values are expressed as ng/g fresh weight (FW) of cells. Data represent the mean ± SD of three independent experiments. (d) The expression of *GST1* (At1g02930), *SAGT* (At2g43820) and *PR1* (At2g14610) was monitored by semi-quantitative, two-step, reverse transcriptase-polymerase chain reaction (RT-PCR). The expression of the *GapC* gene (At3g04120) was used to control an equal amount of cDNA in each reaction (Kato *et al.*, 2003). Primer sequences and PCR conditions are available on request. Similar results were obtained in two additional experiments.

We determined ROS levels by 2′,7′-dichlorodihydrofluorescein diacetate staining (Krause and Durner, 2004), total SA levels by high performance liquid chromatography (HPLC) (Verberne *et al.*, 2002) and cell death by Evans blue staining (Levine *et al.*, 1994). To define the maximal time point for the analysis of these responses, we first assessed cell death timing. Cell death was detected in Pst-AvrRpm1-treated cultures from 8 hpi, and was enhanced until 24 hpi (Fig. 1b). At this time, almost 70% of Pst-AvrRpm1-treated cells were non-viable (Fig. 1b,c), and cultures became white-brown (not shown). In contrast, cell death was not stimulated by Pst-vir during the initial 24 hpi (Fig. 1b,c), and inoculated cultures remained green over this period (not shown). By 36 hpi, dead cells started to be detected in Pst-vir-treated cultures (not shown), probably as a result of its necrotrophic growth phase. Therefore, all subsequent studies were performed during the initial 24 hpi.

Extracellular alkalinization, perceived as a net change in extracellular pH, was induced by Pst-AvrRpm1 from 4.5 hpi, whereas unaltered pH was found in mock- or Pst-vir-inoculated cell cultures (Fig. 2a). The timing and specificity of this response were similar to those *in planta* (Atkinson *et al.*, 1985).

We explored the kinetics of the pathogen-induced oxidative burst before the generation of massive cell death. Pst-AvrRpm1 stimulated sustained ROS accumulation between 5 and 13 hpi, with maximal ROS levels at 10 hpi (Fig. 2b). In contrast, Pst-vir and mock inoculations produced only a minor increase in ROS

levels, which also took place between 5 and 13 hpi. Then, a robust and sustained increase in ROS levels was specifically triggered by the avirulent bacteria. This response resembles the 'phase II' oxidative burst described for incompatible interactions (Lamb and Dixon, 1997). Interestingly, the typical two-phase kinetics of the oxidative burst associated with race-specific resistance in other *in vitro* models (Glazener *et al.*, 1996; Lamb and Dixon, 1997) was not fully reproduced here. The 'phase I' burst, classically induced by elicitors, virulent, avirulent and saprophytic bacteria, was absent in the large majority of our experiments (Fig. 2b), and only detected in a few of them, including Pst-vir or Pst-AvrRpm1, at 2–3 hpi (not shown). This early burst was also absent in other incompatible interactions (Glazener *et al.*, 1996). Although, in this system, we cannot discount early bursts taking place in less than 1 h, it is possible that the phase I burst is repressed by TTSS functions. Such functions may be exacerbated here by the long pre-exposure of pathogens to minimal medium. Results described later reinforce this possibility.

We next analysed SA accumulation in response to pathogens. Total SA levels were quantified in untreated and mock-, Pst-vir- and Pst-AvrRpm1-treated cells isolated at 3, 6 or 9 hpi. The content of total SA in untreated cultures was about 30 ng/g fresh weight of cells (Fig. 2c). Treatment with Pst-AvrRpm1 stimulated two- and 10-fold increases in total SA at 6 and 9 hpi, respectively. In contrast, neither Pst-vir nor mock inoculations altered the basal hormone levels.

In addition, we evaluated the expression of defence genes. As a ROS-responsive gene, we selected *GST1* (glutathione *S*-transferase) (Alvarez *et al.*, 1998). As SA-sensitive genes, we analysed *PR1* and *SAGT* [uridine diphosphate (UDP)-glucosyltransferase; early SA-responsive nonexpresser of PR genes 1 (NPR1)-independent gene; Blanco *et al.*, 2005]. As shown in Fig. 2d, Pst-AvrRpm1 induced *GST1*, *SAGT* and *PR1* from 3–6 hpi. In contrast, Pst-vir treatment did not alter *GST1* or *SAGT* expression, but activated *PR1* by 12–15 hpi. Similar kinetics of RPM1-mediated activation of defence genes were found *in planta* (de Torres *et al.*, 2003).

To examine features of basal resistance, we treated cultures with the non-pathogenic bacteria Psp and Pst-hrpL$^-$. *Arabidopsis* is a non-host plant for Psp and does not generate HR in response to the bean pathogen. Resistance to Psp involves multilayered basal defence signalling, leading to callose deposition associated with papillae formation, among other responses (Ham *et al.*, 2007). *Arabidopsis* neither generates HR nor develops disease in response to Pst-hrpL$^-$ (Zwiesler-Vollick *et al.*, 2002). TTSS from Pst delivers more than 30 effectors in *Arabidopsis* cells, including AvrRpm1 (Alfano and Collmer 2004; Hauck *et al.*, 2003; Kim *et al.*, 2005; Lindeberg *et al.*, 2006; Speth *et al.*, 2007). AvrRpm1 suppresses basal defences contributing to resistance against wild-type and TTSS-deficient Pst, such as callose deposition (Kim *et al.*, 2005).

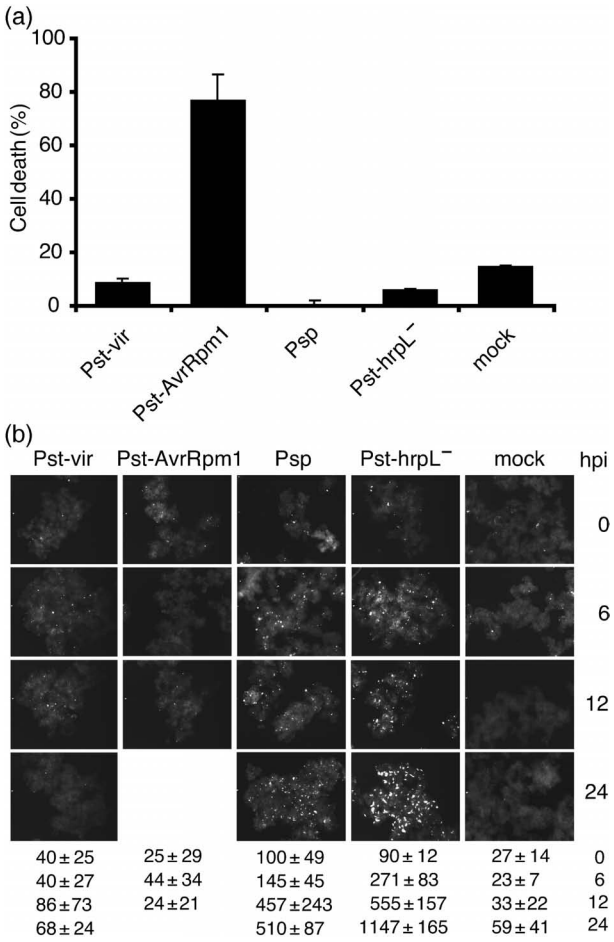

Fig. 3 Features of basal defence in *Arabidopsis* cultured cells. Inoculations were performed as indicated in Fig. 1. (a) Cell death was assayed by Evans blue at 24 h post-inoculation (hpi) as described in Fig. 1. Pst-AvrRpm1 and Pst-vir were used as positive and negative controls, respectively. Data are represented as the mean ± standard deviation (SD) of three independent experiments. (b) Callose deposition in aliquots of inoculated cultured cells. Cells were cleared and stained with aniline blue as described previously (Hauck *et al.*, 2003). Samples were analysed by fluorescence microscopy with an emission filter for 4′,6-diamidino-2-phenylindole (DAPI) (top). The number of callose deposits per cultured cell area (mm^2) was quantified by analysing 8–10 representative pictures from each sample using ImageJ software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). Values represent the mean ± SD (bottom). Similar results were obtained in three independent experiments. Callose deposition was not evaluated in cultures exposed for 24 h to Pst-AvrRpm1 because of massive cellular collapse.

Neither Psp nor Pst-hrpL$^-$ displayed significant proliferation in inoculated cultures during the initial 24 hpi (Fig. 1a). In addition, neither Psp nor Pst-hrpL$^-$ stimulated plant cell death (Fig. 3a). Therefore, in this system, both bacteria behave as *in planta* (Lu *et al.*, 2001; Zwiesler-Vollick *et al.*, 2002).

Early activation of an oxidative burst and callose deposition were evaluated as markers of basal resistance in the challenged

cultures. Callose deposits were quantified from samples stained with aniline blue as described previously (Hauck *et al.*, 2003). Psp and Pst-hrpL$^-$ activated ROS accumulation between 1 and 5–6 hpi, with maximum ROS levels around 2–3 hpi (Fig. 2b). This response was faster and weaker than that stimulated by Pst-AvrRpm1 (Fig. 2b). Therefore, in this system, pathogen-induced ROS enhancement may occur in more than one phase. The kinetics of the oxidative burst triggered by Psp or Pst-hrpL$^-$, and its sensitivity to different microbe-associated molecular patterns, suggest its association with basal defences.

Finally, callose deposition on cell walls was assessed. Cells treated with Psp or Pst-hrpL$^-$ displayed callose accumulation at 6, 12 and 24 hpi (Fig. 3b). In contrast, cells treated with Pst-vir or Pst-AvrRpm1 did not induce this response.

The lack of both the early burst and callose deposition in treatments with wild-type Pst (Figs 2b,3b) indicates the negative modulation of these responses by TTSS functions. Such a negative effect may involve AvrRpm1 and other effectors present in the Pst-vir strain. A reduction in callose deposition is achieved *in planta* by multiple Pst effectors (Hauck *et al.*, 2003; Kim *et al.*, 2005; Speth *et al.*, 2007).

Collectively, our results indicate that the *Arabidopsis* photosynthetic cultured cells recapitulate classical race-specific defences stimulated through RPM1, a coiled coil–nucleotide binding site–leucine-rich repeat (CC-NBS-LRR) protein dependent on non-race-specific disease resistance 1 (NDR1) (Jones and Dangl, 2006). These defences include extracellular pH changes, late and sustained ROS accumulation, SA enhancement, activation of ROS- and SA-responsive genes, and cell death. In addition, the cultured cells respond to non-pathogenic Pst and Psp strains by inducing basal defences, such as an early oxidative burst and callose deposition. Moreover, in this system, as *in planta*, basal defences may be inhibited by Pst, but not Psp, in a TTSS-dependent manner. Thus, this model may constitute a useful tool for the further examination of plant innate immune features, including ROS sources and chloroplast participation in both basal and race-specific resistance, novel networks between the plant defence components, targets of bacterial effectors, among others. The defence responses examined here were stimulated by cells exposed to continuous light, an artificial growth condition. Additional studies will be required to evaluate the effects of light/dark growth regimes on these responses.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Viability of *Arabidopsis* cultured cells. Two aliquots of cultured cells were isolated at each of the indicated time points during the subculturing period. One sample from each pair was maintained without any treatment, whereas the other was incubated at 65 °C for 15 min to heat kill the cells. Both samples were stained with Evans blue to estimate cell death values [optical density at 600 nm (OD_{600}) units]. For each time point, the 100% cell death value is that corresponding to heat-killed cells (squares), whereas the percentage of cell viability indicates the ratio between the cell death values from untreated cells (diamonds) and heat-killed cells. Values represent the mean ± standard deviation from three independent experiments.

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